Project Title: Comprehensive monitoring and mapping antibiotics resistance in orchards

Report Type: Final

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Budget

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Item	2022	2023	2024
Salaries[1]	52,262	54,352	0
Benefits[2]	10,044	10,446	0
Wages			
Benefits			
Equipment			
Supplies[3]	10,342	10,187	0
Travel	5,250	5,250	0
Miscellaneous			
Plot Fees			
Total	**77,898**	**80,235**	**0**

Footnotes: 4% inflation for year 2**.** [1]Postdoc; one PhD and one MSc student, 1.0 FTE, [2]Benefits Postdoc 32.9%; Graduate students 12.6%. [3] Lab and field supplies, antibiotics, plates, primers, gene sequence services, molecular reagents etc, [4]collect samples, April-October.

Objectives:

1. To collect and screen antibiotics (streptomycin, tetracycline and kasugamycin) resistance in apple orchards throughout the state at the population level; -completed
2. To determine the resistance nature (intrinsic or plasmid-borne) of the pathogen if any; -completed
3. To immediately deliver results to growers and provide guidance on antibiotics use in orchards in the coming years.-completed

Significant Findings:

- No *Erwinia amylovora* isolates exhibited resistant to streptomycin and oxytetracycline in 2022, 2023 and 2024;
- 38 and 7 *Erwinia amylovora* isolates exhibited resistance/tolerance to kasugamycin in 2023 and 2024, respectively;
- Among 45 isolates, 73% were isolated from pear samples and 27% from apple samples;
- The resistant/tolerant isolates were isolated from orchards in 10 distinct locations, including Sunnyside, Mattawa, Prosser, Cashmere, Wenatchee, Malaga, and Entiat.
- Minimum inhibition concentration (MIC) against kasugamycin for 186 isolates isolated from different years in Washington state was determined and compared with 141 strains from other states.
- MIC_{50} was higher for WA isolates, and WA isolates from 2024 had the highest MIC_{50} as compared to previous years.
- No mutation was found in the kasugamycin target *ksgA* gene in most of the resistant/tolerant *E. amylovora* isolates except three.
- This is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere.
- These results suggest that growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease.
- Based on our findings, we recommended that growers should mix kasugamycin with oxytetracycline or be in rotation with streptomycin for fire blight control.

Significance to the industry and potential economic benefits. Since the identification of streptomycin-resistant strains of *E. amylovora* by Loper et al. in 1991, there has been limited data in evaluating the status of antibiotic resistance throughout the central Washington regions. The significance of this research to the industry lies in two aspects. First, this is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere and the isolates were from orchards in 10 different locations, including Sunnyside, Mattawa, Prosser, Cashmere, Wenatchee, Quincy, Malaga, and Entiat. These results suggest that resistance may be more widespread than this study has indicated and resistance level has increased in 2024. Growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease. Based on our findings, growers should take precautions in only applying kasugamycin to treat fire blight or should mix kasugamycin with oxytetracycline if still want to use kasugamycin for fire blight control or be in rotation with streptomycin. In summary, the findings of the current project directly benefit the growers of Washington state by providing instant feedback to growers in antibiotics resistance situation in orchards and growers should take immediate actions to avoid control failure.

Methods and Procedures:

In 2022 to 2024, we either collected symptomatic samples in central Washington by our own field trips to local area growers or samples were sent to us via mail by growers or consultants or extension specialists. We also collected asymptomatic blossom samples. Samples were placed in plastic bags and held on ice or in a refrigerator until they were processed. Samples were processed by cutting into small pieces with a sterile knife, washed briefly with sterile water, soaked in 900 µl 10 mM PBS, vortexed, and streaked for isolation onto five types of media: LB, CCT, LB + Sm 100 µg/mL, LB + Kg 100 µg/mL, LB + Tc 20 µg/mL and incubated at 82.5 Fº (28 °C) for 48 - 72 h. Colonies that appeared purple in color on CCT media, smooth, slightly raised and nonfluorescent were suspected to be *E. amylovora*. Screening for resistance was performed by observing the presence of individual colonies on antibiotic media. Isolates of known resistant *E. amylovora* strains were obtained from culture collections for use as positive controls. Isolates were then confirmed by PCR using *E. amylovora* specific primers G1-F and G2-R.

Spot dilution test was performed for selected resistant/tolerant strains (**Figure 1**). Bacteria were grown on LB plates and a single colony was inoculated in LB broth and grown for 24 hr with shaking at 250 rpm. Bacterial suspensions were adjusted to an absorbance of OD_{600} = 1 in PBS and 10-fold serial dilution was made in PBS. For each dilution, 5 µL was spotted onto plates: LB and LB + Kg 50, 75, 100 125, and 150 µg/mL and incubated at 82.5 Fº (28°C) for 48 - 72 h. Bacterial growth was visually observed on plates with or without antibiotics. Growth on plates without antibiotics was used as a control to compare to the plates with antibiotics.

In addition, the minimum inhibitory concentration (MIC) for a total of 327 isolates was determined (**Table 1**). Bacteria were grown on LB plates and a single colony was inoculated in LB broth with shaking at 250 rpm. Overnight bacterial suspensions were adjusted to an initial concentration of OD600 = 0.1 and 2-fold serial dilutions were performed, starting with LB + Kg1000 µg/mL and ending with LB + Kg 0.976 µg/mL. IC_{50} was defined as the concentration of antibiotics at which growth of the bacterium was 50 % less of that of the control without antibiotics. IC_{95} was defined as the concentration of antibiotics at which growth of the bacterium was 95 % less of that of the control without antibiotics.

Selected resistant/tolerant *E. amylovora* isolates were used to amplify the kasugamycin target *ksgA* gene by primers KsgA-F and KsgA-R. PCR products were then sequenced by Eton Biosciences Inc, San Diego, CA and compared to those of known sensitive strains.

Results and Discussion:

Samples were collected from more than 20 apple and pear varieties in central Washington, including 15 apple and 5 pear varieties, i.e. Gala, Jazz, Pink Lady, Fuji, Crips Pink, Granny Smith, Cosmic Crisp, Honey Crisp, Envy, Ambrosia, Golden Russet, Macintosh, and Sweet Tango; Bosc, Anjou, Bartlett and Star Krimson. A total of 186 *E. amylovora* isolates were obtained and confirmed by PCR. Among them, 38 and 7 isolates collected in 2023 and 2024 were shown to be resistant or tolerant to kasugamycin at 100/125 ppm, respectively, where 73% were isolated from pear samples and 27% from apple samples. Among the pear and apple samples, 58% were varieties Bosc or Bartlett and 58% were Pink Lady or Jazz, respectively. However, no isolates were found to be resistant to streptomycin or oxytetracycline.

Among the resistant/tolerant isolates, colony size was significantly smaller as compared to growth of the same isolate on LB medium and spot dilution assay showed similar growth for resistant/tolerant isolates at LB with antibiotics and without antibiotics (**Figure 1**). These resistant/tolerant isolates were ablet to grow on plates with kasugamycin at 50, 75, 100, and 125 ppm. These findings indicated that these isolates from 2023 and 2024 were shown to be resistant/tolerant to kasugamycin.

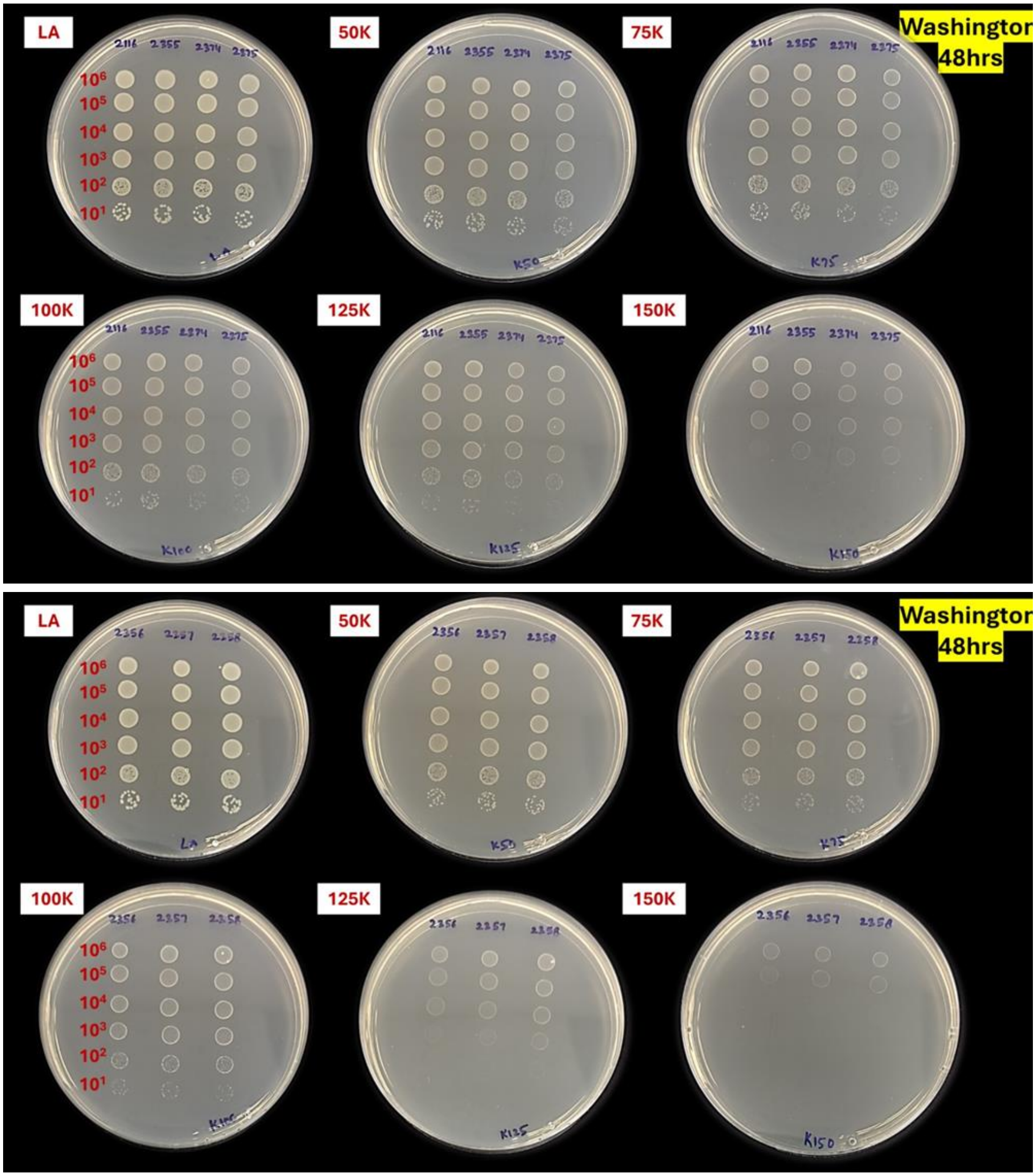

Figure 1. Spot dilution assay for representative resistant/tolerant isolates. Serial 10-fold dilutions were made in PBS. For each dilution, 5µL was spotted on LB plates containing no antibiotics or kasugamycin at 0, 50, 75, 100, 125, and 150 µg/ml. Pictures were taken 48 hours post inoculation.

Next, we determined MIC_{50} and MIC_{95} for 327 isolates from WA or other states. Among them, 186 isolates were collected in different years in WA and 141 isolates were isolated from other states or countries (**Table 1**). The MIC_{50} and MIC_{95} for selected isolates from WA isolated in recent years were shown in **Figure 2** and **Figure 3**, respectively. A few strains isolated from 2024 had the highest MIC_{50} of about 150 µg/ml and the MIC_{50} for most resistant/tolerant isolates was above 60 µg/ml (**Figure 2**). Similarly, a few strains isolated from 2024 had the highest MIC_{95} of about 400 µg/ml and the MIC_{95} for most resistant/tolerant isolates was above 200 µg/ml (**Figure 3**). For the rest of discussion, we only focused on MIC_{50}. By comparing to isolates from other regions or states, the average MIC_{50} for isolates from WA state was the highest as compared to other regions (**Figure 4**) and states/countries (**Figure 5**). In addition, the average MIC_{50} for isolates from WA state in 2024 was the highest as compared to other years (**Figure 6**). Surprisingly, a few strains collected in 2017 and 2018 also showed very high average MIC_{50} value (**Figure 6**) when fire blight epidemic occurred in those years, indicating that strains in WA may start to show resistance to kasugamycin earlier than this report has found.

Sequence comparison of the *ksgA* gene showed no difference for most resistant/tolerant isolates except for three, which had an IC_{50} value above 60 µg/mL, as compared to known type strains (data not shown). Based on previous studies, resistance to kasugamycin arises from mutations of its target gene *ksgA*, encoding an adenine demethylase, or present of a kasugamycin acetyltransferase gene aac(2′)-IIa, which acetylates kasugamycin. Our results indicate that resistance to kasugamycin of most *E. amylovora* isolates except three is not due to mutations in the *ksgA* gene.

Table 1 Numbers of *E. amylovora* isolates used to determine MIC_{50} to kasugamycin

Regions	State/Country	Number of isolates
Northwest	WA	186
West (Northwest)	CA, Utah, Idaho, Oregon, Montana	27
East	New York, Connecticut	88
Midwest	Michigan, Illinois	11
Southeast	Tennessee, Luisiana, Virginia	10
Others	New Zealand, Canada	5

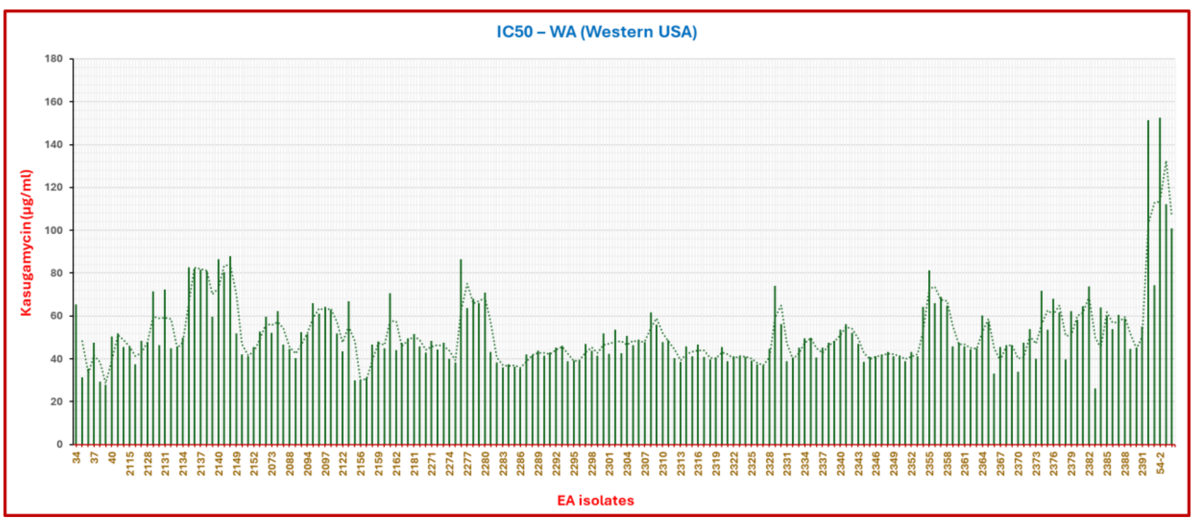

Figure 2 Minimum Inhibition Concentration (MIC_{50}) of selected *Erwinia amylovora* isolates from Washington state against kasugamycin. MIC_{50} was defined as the concentration of antibiotics at which growth of the bacterium was 50 % less of that of the control without antibiotics.

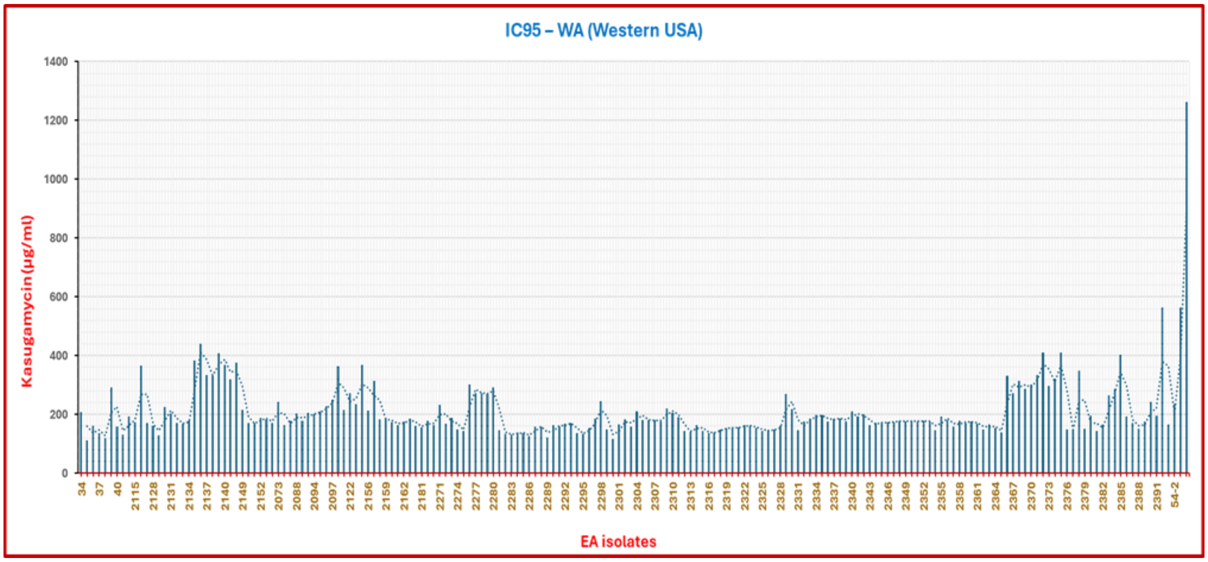

Figure 3 Minimum Inhibition Concentration (MIC_{95}) **of selected *Erwinia amylovora* isolates from Washington state against kasugamycin.** MIC_{95} was defined as the concentration of antibiotics at which growth of the bacterium was 95 % less of that of the control without antibiotics.

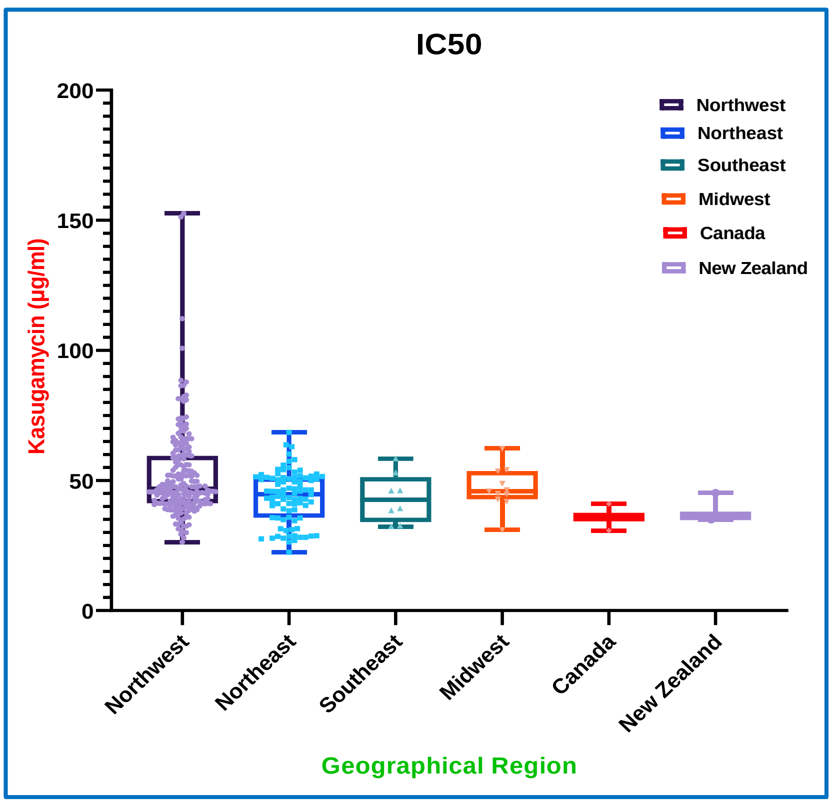

Figure 4 Distribution of minimum Inhibition Concentration (MIC_{50}) **of *Erwinia amylovora* isolates from different regions against kasugamycin.**

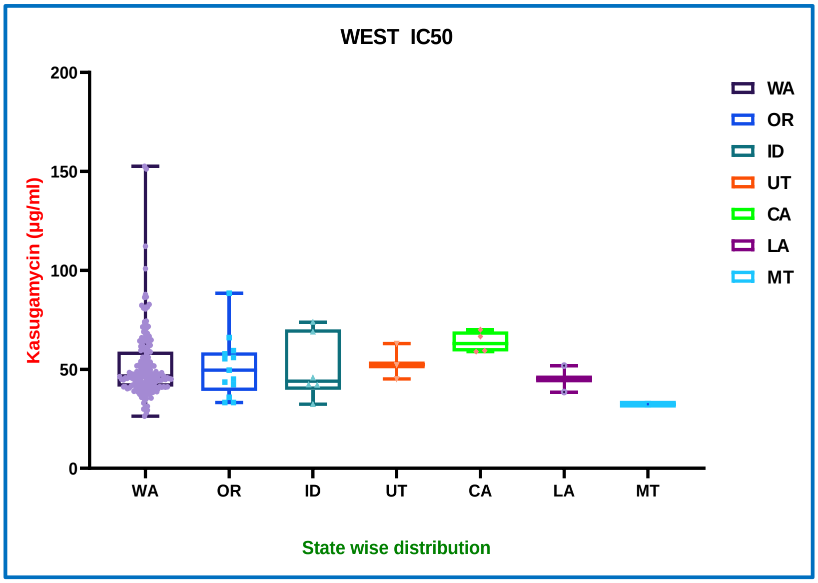

Figure 5 Distribution of minimum Inhibition Concentration (MIC_{50}) of *Erwinia amylovora* isolates from different states against kasugamycin.

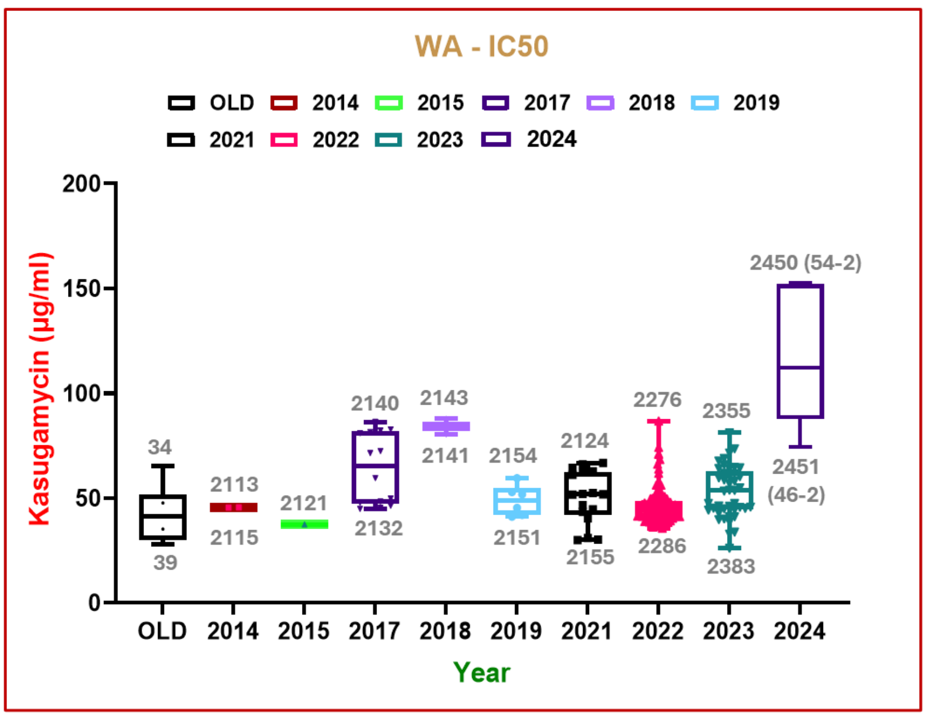

Figure 6 Distribution of minimum Inhibition Concentration (MIC_{50}) of *Erwinia amylovora* isolates from WA isolated in different years against kasugamycin.

Executive Summary

Project Title: Comprehensive monitoring and mapping antibiotics resistance in orchards

Key words: Fire blight, antibiotics resistance, streptomycin, tetracycline, kasugamycin

Abstract: Antibiotics remain one of the best tools for managing blossom blight of apple and streptomycin remains the better choice in terms of cost and efficacy in killing pathogens as compared to tetracycline and kasugamycin. The occurrence of streptomycin resistance of the fire blight pathogen in WA pear orchards in 1980s results in increased use of tetracycline and kasugamycin. However, there has been limited data evaluating the existence and extent of antibiotic resistance of *Erwinia amylovora* in central WA since then. The purpose of the current study was to comprehensively monitor and map antibiotics resistance in orchards in WA. During the 2022 to 2024 growing seasons, hundreds of diseased samples were collected from apple and pear orchards and 186 *E. amylovora* isolates were examined for their resistance to streptomycin, oxytetracycline and kasugamycin. Although no *E. amylovora* isolates exhibited resistance to streptomycin and oxytetracycline, 38 and 7 isolates exhibited resistance or tolerance to kasugamycin in 2023 and 2024, respectively. Among them, 73% and 27% were isolated from pear and apple samples, respectively. Minimum inhibition concentration (MIC) for these resistant/tolerant isolates was compared with 141 strains from other states and from different years. MIC_{50} was higher for WA isolates, and WA isolates from 2024 had the highest MIC_{50} as compared to previous years. No mutation was found in the kasugamycin target *ksgA* gene in most of the resistant/tolerant *E. amylovora* isolates except three. This is the first report of kasugamycin resistant/tolerant *E. amylovora* isolates in Washington or elsewhere and the isolates were from orchards in 10 distinct locations, including Sunnyside, Mattawa, Prosser, Cashmere, Wenatchee, Malaga, and Entiat. These results suggest that growers should take immediate actions in terms of how to and what antibiotic to use for controlling fire blight disease. Based on our findings, we recommended that growers should mix kasugamycin with oxytetracycline or be in rotation with streptomycin.